SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the effects of cardiac myosin activators on systolic and diastolic function *in vitro* and *in vivo*

by Arnold Péter Ráduly, MD

Supervisor: Attila Borbély, MD, PhD

UNIVERSITY OF DEBRECEN

KÁLMÁN LAKI DOCTORAL SCHOOL

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Kálmán Laki Doctoral School, University of Debrecen

Head of the Defense Committee: Csongor Kiss, PhD, DSc
Reviewers: Róbert Sepp, PhD, DSc
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1 Introduction

1.1 Definition, epidemiology, and classification of heart failure

Heart failure (HF) is a complex clinical syndrome resulting from structural and/or functional damage to the heart, in which the patient has the typical symptoms (shortness of breath, dyspnoea, fatigue) and signs (distended jugular veins, pulmonary congestion, ankle oedema) of heart failure. HF is very common in both developed and developing societies due to ageing and evolving therapies.

According to the 2021 European Society of Cardiology (ESC) professional guideline, the classification of HF is based on the left ventricular ejection fraction (LVEF): 1. HF with reduced ejection fraction (HFrEF, LVEF<40%), 2. Heart Failure with mildly reduced Ejection Fraction (HFmrEF, LVEF 41-49%) and Heart Failure with preserved Ejection Fraction (HFpEF, LVEF≥50%).

1.2 Pathophysiology and pharmacological treatment of systolic heart failure (HFrEF)

Regardless of the aetiology, the central feature of HFrEF is the contractile dysfunction and consequent reduction in cardiac output (CO), which triggers several mutually reinforcing pathophysiological processes. The CO is the product of heart rate (HR) and stroke volume (SV). The factors that determine SV are preload, afterload, and contractility. The contractility characterizes the inotropic state of the heart. The compensatory mechanisms activated in heart failure are in fact an attempt to normalise/restore the reduced CO and the reduction in mean arterial pressure. These are the Frank-Starling mechanism, pathological activation of the neurohumoral system and LV remodelling. The general feature of the disease modifying drugs that they can reduce mortality and hospitalizations, thus improving the prognosis of the disease. Among the renin-angiotensin-aldosterone system inhibitors, angiotensin-convertase enzyme inhibitors and angiotensin receptor blockers (in case of intolerance) have particular importance. Angiotensin receptor neprilysin inhibitors (ARNIs), which also affect the natriuretic peptide pathway, has a lot of evidence on its beneficial effects in HFrEF. Overactivation of the sympathetic nervous system can be decreased by selective β1-receptor blockers. Mineralocorticoid receptor antagonists (MRAs) can also be used with good efficacy, as they can effectively enhance sodium excretion through inhibition of sodium reabsorption. As a result of intensive research and clinical studies in recent years, the use of sodium glucose cotransporter 2 (SGLT2) inhibitors has also been incorporated into the therapy of HFrEF.

1.3 Advanced heart failure

Heart failure is a progressive disease that leads to a gradual deterioration in the quality of life. The New York Heart Association (NYHA) distinguishes four functional stages (NYHA I-IV) based on daily activity and subjective complaints of the patients. We are speaking about advanced heart failure if the patient's condition worsens despite the optimal pharmacological and device therapy. Further therapeutic considerations are required in this population. The definitive, permanent solution is heart transplantation or, in some patients, mechanical circulatory support. However, as few patients meet the strict criteria, a higher proportion of patients will be treated conservatively/pharmacologically with positive inotropic/inodilator drugs. Long-term use of these agents has been shown in several studies to increase mortality and have neutral effect on hospitalization rate. Current research aims at developing selective contractility-enhancing drugs that can be used at an earlier stage of HFrEF and that primarily target the contractile dysfunction to achieve the improvement of cardiac contractility.

1.4 Classification of positive inotropic agents

1.4.1 Drugs with conventional or upstream mechanism

Agents that act via an ’upstream’ mechanism include conventional positive inotropic agents that affect intracellular calcium concentrations through modification of various signal transduction pathways. The members of this group are the traditional positive inotropic agents: dobutamine, used in clinical practice mainly in the treatment of acute HF and it can induce phosphorylation processes through the activation of β1-receptors, which ultimately lead to an increase in intracellular calcium levels. Other members of the group are dopamine, milrinone, adrenaline, noradrenaline, and cardiac glycosides. A common feature of these drugs is increasing myocardial energy demand and oxygen consumption because of increasing calcium levels. These effects make these agents highly detrimental to myocardial energetics. Furthermore, intracellular calcium overload is a nest for the development of atrial and ventricular arrhythmias.

1.4.2 Agents with central and downstream mechanism of action

Drugs with a 'central' mechanism, such as levosimendan (Levo), increase the affinity of TnC for Ca^{2+}. Moreover, Levo induces vasodilation through activation of the ATP-sensitive K^{+} channel, hence they are also called inodilators. Levo also shows the characteristics of upstream mechanism, as it has a PDE3 inhibitory effect at higher plasma concentrations.

The 'downstream' mechanism is characterised by no effect on IC Ca^{2+} levels, thus completely free of the side effects of conventional positive inotropic agents. These agents could be energetically beneficial for myocardial cells. Currently, molecules belonging to this group act through enhancing myosin-ATPase activity. An earlier member of this group is EMD 53998 (EMD), which is an enantiomeric molecule. One pair acts via downstream mechanism, while the other has PDE3 inhibitory activity. Intensive research has resulted in the development of omecamtiv mecarbil (OM) and danicamtiv in the last decade, which in fact act only by enhancing the ATPase activity of the myosin molecule, hence they are referred to as myosin activators.

1.5 Myosin activators

1.5.1 Mechanism of action

The sarcomere is the basic molecular unit of myocardial contraction, which is made up of a combination of thin (actin) and thick (myosin) filaments. The myosin molecule is composed of two globular heads and a tail domain. The myosin head has a paramount importance because it contains the actin and adenosine triphosphate (ATP) binding site and has ATPase activity, thus being able to convert chemical energy into physical energy. The myosin activators - OM and danicamtiv - can selectively bind to the S1 region of cardiac type myosin and enhance its ATP-hydrolysing capacity. This promotes the formation of ADP and P_i, and the dissociation of P_i from the myosin head, thus accelerating the rate-determining step of the actomyosin cycle and the formation of the myosin-ADP complex. They facilitate the conversion from the weakly-bound form to the strongly bound form, followed by the force generation step. Theoretically, this process increases the number of actin-myosin interactions that occur during a cycle, therefore an increase in force production takes place without an increase in oxygen consumption.

1.5.2 Omecamtiv mecarbil

The development of anti-tumor therapy has involved the search for molecules that could inhibit the motor proteins (kinases) involved in cell division, using a high-throughput technique (HTS). However, they found a molecule which activates the motor proteins rather than an inhibiting one. Further modifications led to Omecamtiv mecarbil (OM) (CK-1827452), which proved to be suitable for *in vivo* experiments and was effective in enhancing the activity of myosin ATPase. Preclinical studies demonstrated its ability to enhance the contractile force of myocardial cells isolated from rat. OM slightly prolonged the contraction time, without

affecting the calcium transient. *In vivo* studies in various animal models of HF revealed improvement in left ventricular function without an increase in oxygen consumption.

Positive results from *in vivo* and *in vitro* studies allowed the researcher to design human trials with the new drug candidate. The first dose-escalating clinical trial and the ATOMIC-HF study compared iv. OM treatment with placebo. Later, the COSMIC-HF study applied per os treatment. These studies predominantly recruited HFrEF patients with an EF below 40%. The improvement in systolic function, the significant prolongation of SET in these studies and the good tolerability and safety of the drug were highlighted. No differences were found in adverse events, although a small increase in TnI levels was observed, but this was not associated with the dose of OM used in the ATOMIC-HF study. In the GALACTIC-HF phase III clinical trial. The primary composite endpoint, i.e., CV death or event due to HF (hospitalization or emergency visit), was significantly lower in OM-treated patients compared to the placebo group. When the components of the composite endpoint were examined separately, there was no significant difference in CV mortality, but a significant reduction in HF events was observed in the OM group. There was also a small but non-significant increase in TnI levels and a significant decrease in NT-proBNP levels in OM-treated patients.

1.5.3 Danicamtiv

The ambiguous results of clinical trials with OM have prompted researchers to develop a new direct myosin activator, the danicamtiv. Like OM, danicamtiv accelerates the rate of ATPase activity of purified human S1 myosin molecules *in vitro* and increases SET and LVEF, as well as left atrial systolic function in an *in vivo* experimental dog model of HFrEF. A phase I clinical trial demonstrated the safety of danicamtiv, based on which results were designed and dose-escalation studies were conducted in heart failure patients, where a transient asymptomatic increase in Tn plasma concentrations was observed in 23% of patients with improvement in LV systolic function. In a recent preclinical study, a wider therapeutic window, greater contractile function improvement potential and a more favourable lusitropic effect were associated with danicamtiv compared to OM.

2 Aims

We aimed to investigate the effect of different OM concentrations in canine cardiomyocytes, *in vitro* and to analyse the changes in systolic and diastolic sarcomere length, their kinetics and

correlations, and the changes in IC Ca^{2+} transients. EMD and Levo were used as comparator compounds.

In our *in vivo* study we assessed the effects of the new type of myosin activator, danicamtiv on cardiac cycle, systolic and diastolic function.

3 Materials and Methods

3.1 In vitro experiments

3.1.1 Isolation of canine cardiomyocytes

Canine cardiomyocytes were studied since their electrophysiological properties are similar to those of humans. Briefly, hearts were isolated from anesthetized (ketamine-HCl 10 mg/kg, Richter Gedeon, Hungary, xylazine-HCl 1 mg/kg, Eurovet Animal Health BV, The Netherlands) adult mongrel dogs (N=6) weighed 10.6±2.1 kg and aged 15.1±4.2 months (10.9 to 23.9 months). Thereafter, the hearts were subjected to a perfusion system where the left anterior descending coronary artery was cannulated. Single cardiomyocytes were obtained by enzymatic dispersion technique. Cardiomyocytes were stored at 15°C until the measurements.

3.1.2 Measurement of Ca^{2+} level changes and sarcomere length shortening

Cardiomyocytes were loaded with 5 μM Fura-2 AM Ca^{2+} sensitive ratio metric fluorescent dye for 30 min in the presence of Pluronic F-127 (25 mg/ml) to avoid early elimination of the dye from the intracellular space. Twenty-five milligrams Pluronic F-127 was dissolved in 1 ml DMSO and this solvent was used to make a Fura-2 AM stock solution. Cells were then incubated for 30 min to allow the intracellular esterases to release Fura-2. Cardiomyocytes were placed in a chamber on the stage of an inverted microscope (Nikon TS-100). The final volume of the chamber filled with Tyrode solution (containing 144 mM NaCl, 5.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, and 11 mM dextrose, pH=7.4) was 1 ml. After sedimentation a rod-shaped cardiomyocyte with clear striation, and acceptable contraction upon field-stimulation was selected for further experiments. Field stimulation was performed at 0.1 Hz (Experimenta Setup, MDE, Heidelbeg). Alternating excitation wavelengths of 340 nm and 380 nm were used to monitor the fluorescence signals of Ca^{2+}-bound and Ca^{2+}-free Fura-2 dye, respectively. Fluorescent emission was detected above 510 nm in case of both wavelengths, and traces were digitized at 120 Hz using the FeliX Software (Ratiomaster RM-50 system, Horiba, New Brunswick, NJ, USA). The experimental protocol was the following:

cardiomyocytes were paced at 0.1 Hz for at least 2-3 min to achieve a steady-state at the beginning of each experiment. The resting (unstimulated) SL was continuously measured after this initial conditioning using a high-speed camera. OM (with final concentrations of 0.03 μM, 0.1 μM, 0.3 μM or 1 μM), Levo (1 μM), or EMD (1 μM) was added for 5-8 minutes to the experimental chamber.

3.1.3 Characterisation of contractile parameters and Ca^{2+} transients

Contractile responses were monitored at 0.1 Hz pacing rates. Multiple parameters of cardiomyocyte contractions, relaxations and intracellular Ca^{2+} transients were assessed. Resting SL of the cardiomyocytes was measured using a high-speed camera. Fractional sarcomere shortening (FS) was calculated using the following equation: FS=(diastolic SL-systolic SL/diastolic SL)*100. Duration of contraction (DC; s) was defined as the time from the beginning till the end of the contraction of the cardiomyocyte. Rates (μm/s) of contraction and relaxation were determined by linear fits to the apparently linear phases of contractions and relaxations, respectively. The resting Ca^{2+} level was estimated by the Fura-2 ratio (fluorescent intensity ratio at 340 and 380 nm excitations) at baseline (before cardiomyocyte stimulation). The amplitude of Ca^{2+} transients was defined as the difference between the peak levels of Ca^{2+} transients and resting Ca^{2+} levels (340 nm/380 nm ratio). The rate of Ca^{2+} transient increase was considered as the slope of a line fitted by linear regression to the ascending phases of Ca^{2+} transients (1/s). The Ca^{2+} transient decay kinetics was fitted to a single exponential and described by its rate constant (K; 1/s).

3.2 *In vivo* experiments

3.2.1 Experimental setup, animal model

In vivo experiments were performed on 8-12-week-old adult Sprague-Dawley rats (417.90±51.95 g, Charles River Laboratories Inc., Germany) and they were housed in a room with controlled temperature and kept under 12/12 h dark/light cycle. Rats were anaesthetized with ketamine/xylazine combination (75/5 mg/kg, respectively); thereafter, chest hair was shaved, tail vein was canulated (the cannula was removed at the end of the experiments), and animals were positioned in a dorsal position on a heating pad (39 °C). During echocardiographic examinations, external 3-lead ECG registration was continuously performed. Danicamtiv was administered intravenously at a dose of 2 mg/kg according to a protocol approved by the local Animal Care Committee (4-1/2019/DEMÁB).

3.2.2 Echocardiography

Echocardiography was carried out using the Vevo 3100 Imaging System including Vevo Imaging Station (VisualSonics, Amsterdam, The Netherlands) equipped with high-frequency transducer (MX250, 14–28 MHz). Echocardiographic imaging was started 5 min after danicamtiv iv. injection and lasted maximum 15 min. Data acquisition was performed in B-, 2D-, M-, and Doppler modes, from parasternal long- and short axis (PSLAX, PSAX, respectively), as well as suprasternal and apical 4 chamber views (SST, A4C, respectively). Heart rate (HR, bpm) was automatically calculated from the ECG R-R interval data of 5 s. Wall thickness and chamber diameters were measured in M-mode, at mid-level of the papillary muscles, from both PSLAX and PSAX views. End diastolic diameter (EDD, mm) and end systolic diameter (ESD, mm) were measured by manually tracing the endo- and epicardial borders in PSLAX M-mode images. Left ventricle volume in diastole and systole (EDV, ESV, respectively; µL) were calculated by the software as (7.0/(2.4+EDD))*EDD3, and (7.0/(2.4+ESD))*ESD3, respectively. The LV ejection fraction (LVEF, %) was measured as 100*(EDV-ESV)/EDV. Stroke volume (SV, µL) was determined as LVIDd-LVIDs, and Cardiac Output (CO, mL/min) was estimated as SV*HR. To measure kinetics of endocardial wall contraction, M-mode velocity at the LVPW wall (MVel, mm/s) was determined by manually tracing the wall movement on the M-mode images.

Diastolic function was assessed by pulsed-wave Doppler (PWD) and tissue Doppler imaging (TDI) from apical 4 chamber views at the levels of the mitral valve (MV) and the septal annulus, respectively. Transmitral early (MV E, mm/s) and late atrial (MV A, mm/s) peak flow velocities, MV E/A ratio, and deceleration time (DecT, ms) of the E wave were determined. Isovolumic contraction time (IVCT, ms), systolic ejection time (SET, ms), and isovolumic relaxation time (IVRT, ms) were determined in the left ventricular cavity, where both mitral inflow and left ventricular outflow could be visualized. Myocardial performance index (MPI) was calculated as the Tei-index (IVRT+IVCT/LVET(SET)). The length of a cardiac cycle (CL; ms) was determined from R-R distance. Duration of systole (SystDur; ms) was considered as ET+IVCT, while du-ration of systole (DiastDur; ms) was calculated as CL-SystDur. The ratio of the DiastDur/SystDur was also calculated. Tissue Doppler imaging (TDI) was performed at the septal annulus to evaluate peak tissue velocities at systole (s', mm/s), and in early (e' mm/s) and late (a', mm/s) filling. The ratio of E/e' was then determined. Three cardiac cycles were averaged for each parameter.

3.2.3 Strain echocardiography

Strain echocardiographic analysis was carried out offline. During the conventional echocardiographic imaging, high-frame rate (>200 fps) traces were recorded from both the parasternal long- and short axis views (PLAX and SAX, respectively), by a trained investigator. Longitudinal and radial strain parameters were generated from PLAX view, while circumferential strain parameters were assessed from SAX view images, with papillary muscles excluded from tracing. Ventricular borders were defined, and the speckle-tracking system assigned circumjacent areas adjacent to the outlined chamber. Speckle-tracking data were visualized in a color-coded map representing diastolic and systolic deformations and reconstructed in a 3D image to provide a better spatial-temporal perception of wall motion. Peak and average radial systolic velocity values (cm/s), global longitudinal strain (GLS; %) and global circumferential strain (GCS; %) after iv. danicamtiv administration were analysed and compared to baseline values of each animal.

3.2.4 Electrocardiography

Three lead ECG recording was performed in parallel with the echocardiographic examinations. After anaesthesia, animals were placed in a dorsal position and leads were positioned subcutaneously. LabChart Reader v8.1.14 software was used to evaluate ECG recordings. The following parameters were considered for the evaluation: heart rate, PQ interval, QRS duration, QT time, corrected QT interval, T-wave amplitude. For the evaluation, 5 consecutive cardiac cycles were averaged for each parameter.

3.3 Drugs and materials

The OM used in the experiments was ordered from AdooQ BioScience. The levosimendan was provided by our Finnish collaboration partner. For the in vitro experiments, each drug was dissolved in DMSO and a stock solution of 10 µM was prepared and stored at -20°C, from which the final concentrations were diluted, and cells were treated. Other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Danicamtiv was purchased from MedChemExpress (NJ, USA). Stock solutions for *in vitro* experiments were prepared in DMSO as solvent and stored at 4 °C. The concentration of DMSO in these solutions was 0.1%. The control (danicamtiv-free) vehicle contained the same amount of DMSO. Regarding *in vivo* experiments, danicamtiv was administered iv., dissolved in its special solvent (10% DMSO/90% (20% SBE-β-CD in saline)) at a dose of 2 mg/kg. Control experiments were

performed with solutions containing only the vehicle (10% DMSO/90% (20% SBE-β-CD in saline)).

3.4 Statistical analysis

Results were evaluated and graphs were created in the GraphPad Prism 8.0 and 9.0 software (GraphPad Software, San Diego, CA, USA). The number of experiments in each group varied between 6-10 from 6 different hearts. Background fluorescence intensity levels were obtained at the end of the measurements, on a region without cardiomyocytes and were manually subtracted the fluorescence intensities for background correction. Fourteen rats were measured before and after iv. treatment of danicamtiv. Values were evaluated for normality (Kolmogorov Smirnov normality test) and were then evaluated by paired t-tests, ordinary one-way ANOVA or Kruskal-Wallis test with multiple comparisons as appropriate. Group descriptions are given as mean±SEM values. Statistical significance was accepted at $P<0.05$.

4 Results

4.1 *In vitro* experiments - Omecamtiv mecarbil, EMD-53998 and Levosimendan

4.1.1 OM and EMD, but not Levo reduce cardiomyocyte resting sarcomere length

A dose-dependent decrease in the resting SL of cardiomyocytes was observed upon OM administrations (i.e., from the drug free control of 1.96±0.01 μm to 1.94±0.04 μm, 1.77±0.04 μm, 1.62±0.05 μm or 1.50±0.05 μm at 0.03 μM OM, 0.1 μM OM, 0.3 μM OM or 1 μM OM, respectively; SL changes at 0.1 μM OM concentrations and beyond were significant). 1 μM EMD also evoked a significant reduction in resting SL (i.e., to 1.83±0.08 μm), while Levo did not affect resting SL. Intracellular resting Ca^{2+} concentrations remained unchanged during OM, EMD or Levo treatments.

4.1.2 *OM, EMD and Levo differently affected cardiomyocyte contraction and relaxation kinetics*

A significant decrease in the systolic peak SL was observed at 0.03 μM, 0.01 μM, 0.3 μM or 1 μM OM concentrations (1.65±0.03 μm 1.53±0.03 μm, 1.39±0.02 μm, 1.34±0.03 μm, respectively vs. 1.74±0.01 μm measured under control conditions, $P<0.05$ for all) in field-stimulated cardiomyocytes. Similarly to OM, EMD significantly decreased the systolic peak SL (i.e. to 1.51±0.03 μm, $P<0.05$). The effects of Levo on systolic peak SL decreases also reached the significance level (i.e., this parameter decreased to 1.63±0.03 μm, $P<0.05$). OM increased fractional sarcomere shortening at 0.03 μM (i.e., to 14.34±1.64%), but not at higher

drug concentrations while EMD and Levo significantly increased this parameter at 1 μM (i.e., to 17.36±0.98% and 17.02±0.80%, respectively from the drug-free control level of 11.17±0.55%, $P<0.05$). The amplitudes of Ca^{2+} transients were not affected by any of the applied drugs.

A progressive prolongation of contraction time was observed upon increasing OM concentrations (1.50±0.33 s, 2.31±0.21 s, 3.85±0.15 s and 5.89±0.57 s, at 0.03 μM, 0.1 μM, 0.3 μM and 1 μM OM concentrations, respectively vs. 0.95±0.05 s in drug-free controls), while EMD and Levo did not affect this parameter. The prolongation of contractile responses could be attributed to slower kinetics of both contractions (from 0.65±0.05 μm/s to 0.51±0.13 μm/s, 0.27±0.10 μm/s, 0.11±0.02 μm/s and 0.05±0.01 μm/s) and relaxations (from 0.95±0.10 μm/s to 1.12±0.30 μm/s, 0.29±0.06 μm/s, 0.13±0.03 μm/s, 0.05±0.01 μm/s), respectively, with increasing OM concentrations (control, 0.03 μM, 0.1 μM, 0.3 μM and 1 μM, respectively. In contrast to OM and EMD Levo increased the kinetics of both contractions and relaxations. The durations of the Ca^{2+} transients, kinetics of the upstrokes of intracellular Ca^{2+} transients and the kinetics of the Ca^{2+} transient decays (illustrated by the rate constant, K) remained unaffected by all drug treatments.

4.1.3 OM uniquely alters intracellular Ca^{2+}-sarcomere length relationship

To further characterize the relationships between intracellular Ca^{2+} transients and contractile responses sarcomere length was expressed as a function of intracellular Ca^{2+} concentration upon positive inotropic agent administrations. These Ca^{2+}-SL relationships did not largely differ before and after 0.03 μM OM exposures (Fig. 4A). However, following 0.1 μM, 0.3 μM or 1 μM OM administrations loop diagrams markedly and progressively shifted downward, suggestive for a Ca^{2+}-sensitizing effect that was present both at systolic and diastolic intracellular Ca^{2+} concentrations. The effect of 1 μM EMD on Ca^{2+}-SL relationship was similar to that observed in the presence of 0.1 μM OM. Ca^{2+}-SL relationship in the presence of Levo was enlarged towards shorter systolic SLs, consistent with a Ca^{2+}-sensitizing effect that developed during systoles but not during diastoles.

4.1.4 Interactions between diastolic and systolic sarcomere dynamics

To elucidate further the three different drug-target interactions, parameters of systolic responses upon OM, EMD or Levo administrations were expressed as functions of their respective diastolic SLs. This approach verified hypothetical similarities between the OM and EMD dependent effects when interrelations between diastolic SLs and peak systolic SL or contraction

durations were analyzed. Nevertheless, diastolic SL-fractional sarcomere shortening relationships suggested distinctions between OM and EMD. Furthermore, Levo induced systolic parameters apparently did not require changes in diastolic SL, consistently with a Levo induced Ca^{2+} -sensitizer mechanism different from those evoked by OM or EMD.

4.2 *In vivo* experiments - Danicamtiv

4.2.1 Danicamtiv decreases end-systolic diameter but does not affect end-diastolic diameter

A significant decrease in the LV end-systolic diameter (ESD), but no change in the LV end-diastolic diameter (EDD) were observed upon the intravenous administration of 2 mg/kg danicamtiv (EDD: from 7.56±0.12 to 7.74±0.26 mm, P=0.45, ESD: from 3.76±0.11 to 2.93±0.17 mm, P< 0.001) in rats.

4.2.2 Danicamtiv improves left ventricular contractile function in vivo

The relevant LV systolic contractile parameters, including LVEF (from 79.76±1.24 to 89.36±0.99%, P<0.001), fractional shortening (FS) (from 50.58±1.3 to 63.57±1.47%, P<0.001), and stroke volume (SV) (from 243.89±10.07 to 290.56±20.26 µL, P=0.012) increased significantly upon the intravenous administration of 2 mg/kg danicamtiv. In addition, cardiac output (CO) also increased (from 61.40±2.99 to 72.45±5.04 mL/min, P=0.026), supporting the positive inotropic effect of the drug, and the consequent hemodynamic improvement.

4.2.3 Duration of diastole and systole changes as a result of danicamtiv treatment in vivo

Both systolic durations (from 88.64±1.83 to 106±3.3 ms, P=0.0001) and systolic ejection time (SET) were significantly prolonged upon danicamtiv treatments in rats (from 72.28±1.53 to 87.14±3.05 ms, P<0.001) Echocardiography revealed a significant decrease in the duration of diastole (from 152.20±4.19 to 133±4.66 ms, P=0.008), which was also evidenced by a marked decrease in the ratio of diastolic to systolic durations (from 1.73±0.06 to 1.28±0.07, P<0.001).

4.2.4 Contraction kinetics slows down significantly, but left ventricular strain parameters improve after danicamtiv treatment

When danicamtiv was applied to rats, a significant decrease in LV peak radial systolic velocity (from 3.50±0.13 to 2.67±0.17 cm/s, P<0.001, N=9) and contraction velocity (M-mode slope) (from 3.32±0.23 to 2.30±0.11 cm/s, P=0.008, N=10) were observed. Upon danicamtiv administration, both global longitudinal strain (from −24.22±1.30 to −33.27±1.45%, P<0.001)

and global circumferential strain (from −54.58±3.28 to –59.29±3.36%, P=0.015) decreased significantly, thus indicating LV systolic function improvement.

4.2.5 Danicamtiv treatment leads to diastolic dysfunction in vivo

Danicamtiv administration was associated with a significant decrease in the early (E) transmitral filling velocity (from 720.92±11.87 to 645.92±29.17 mm/s, P=0.013) and an increase in the late (A) transmitral filling velocity (from 379.24±19 to 506.93±37.81 mm/s, P=0.003). The consequent decrease in the E/A ratio (from 1.97±0.10 to 1.38±0.10, P=0.0003) indicated an elevation in left ventricular filling pressures. The tissue doppler imaging (TDI) velocities (i.e., mitral valve septal early (a') and late (e') filling tissue velocities) tended to change similarly to the mitral E and A wave velocities. The changes in the TDI e'/a' ratio further support the altered mitral inflow pattern upon danicamtiv administration. Nevertheless, the isovolumic relaxation time (IVRT) was not altered by danicamtiv.

5 Discussion

5.1 Omecamtiv mecarbil exerts positive inotropic effects and induces diastolic dysfunction in vitro

Our data are consistent with an OM-evoked Ca^{2+}-sensitizing effect, whereby its positive inotropic effect develops primarily by prolongation of systolic contractions rather than by major changes in fractional sarcomere shortening or augmentation of the kinetics of cardiac contractions or relaxations. Here we report that OM, EMD and Levo evoke robust changes in cardiomyocyte contractions and relaxations in the absence of significant changes in intracellular Ca^{2+} transients, suggestive for Ca^{2+}-sensitization for all three agents. Nevertheless, the characteristics of these Ca^{2+}-sensitizing mechanisms were different for OM, EMD and Levo, and hence resulted in different kinds of contractile responses. Although both Levo and EMD has inhibitory effect on PDE III, the unchanged intracellular Ca^{2+} levels were somewhat surprising in the presence of these agents and can be potentially explained by PDE isoforms (other than PDE III) not inhibited by these agents in canine cardiomyocytes at the employed drug concentrations.

OM evoked a reduction in diastolic SL of unstimulated cardiomyocytes of dog hearts at low intracellular Ca^{2+} concentrations. The magnitude of this decreases in SL was comparable to that observed in stimulated cardiomyocytes during diastoles, suggesting that the OM-dependent activation of the actin-myosin interaction did not require Ca^{2+}. These findings are also in accord with the OM-stimulated increase of basal myosin ATPase activity of rabbit hearts

and isometric force production in permeabilized cardiomyocytes of rat hearts at diastolic Ca^{2+} levels. Similarly to OM, EMD but not Levo decreased resting SL, revealing similarities for the two myosin-binding agents and a distinct effect for the thin filament selective Levo in their Ca^{2+}-sensitizing effects. Here we also show that the reductions in diastolic and systolic SLs, and contraction durations are tightly coupled to the applied OM concentrations, and thus illuminate different facets of the same drug-target interaction on the myosin molecule. The reduction in diastolic SL was probably also responsible for the unchanged fractional sarcomere shortenings in the presence of high OM concentrations despite the observed reductions in peak systolic SLs. Interestingly, in an independent study, fractional cell shortening did increase by OM in rat cardiomyocytes, nevertheless, based on our results, we propose that the increase in fractional cell shortening can be limited by the OM evoked decrease in resting SL at higher OM concentrations.

The reduction in resting SL increased in a range of OM concentration (i.e., between 0.01 μM and 1 μM) overlapped with that reported during its clinical administrations. In the most recent clinical trial of OM (GALACTIC-HF) a guided dose titration strategy was applied to achieve plasma concentrations of at least 200 ng/ml (0.5 μM), while avoiding concentrations >1.000 ng/mL (2.5 μM). It is important to note, that OM concentrations >1200 ng/ml (3 μM) (three times higher than the maximum concentration used in our in vitro experiments) were previously reported to lead to excessive prolongation of the systole, thus limiting coronary blood flow during diastole, and possibly leading to myocardial ischemia. In animals and humans, the pharmacodynamic signature of OM is an increase in the systolic ejection time (SET). This observation is the reflection of the drug's most significant mechanism of action, as contractile activity can be maintained by OM even when cytoplasmic Ca^{2+} concentration falls. Despite the slower kinetics of force generation in *vitro*, the maximal rate of LV pressure development (dP/dt_{max}) was shown to be unaffected by OM *in vivo*, suggestive for distinct pharmacokinetic properties for OM in vitro and *in vivo*.

The durations of cardiomyocyte contractions increased almost six times at high OM concentrations, implicating a prolonged activation for the contractile protein machinery due to a delay of the inactivation of the thin filaments and increased number of strongly attached cross-bridges. The increase in the half-time of activation ($t_{1/2}$ of activation) and the decrease in the rate constant of force redevelopment (k_{tr}, illustrating the intrinsic kinetics of the actin-myosin cross-bridges) observed in our previous study in permeabilized rat cardiomyocytes are also in line with our present observations and corroborate the decreased in vitro motilities of the

myosin filaments. Taken together, OM-evoked Ca^{2+}-sensitization may contribute to stronger, slower, and prolonged cardiac contractions consistently with previous echocardiographic findings.

Of note, the OM evoked increase in contraction durations was not observed during EMD administrations, and this might be a consequence of their distinct binding sites on the myosin molecule. The binding site of OM on the myosin S1 domain probably resides in a cleft in the vicinity of its actin-binding interface and of the nucleotide binding pocket. This location is supposedly ideal for allosteric modulation of both the enzymatic and mechanical properties of the cardiac myosin motor. The EMD binding site is not identical with that of OM on the myosin S1 domain, nevertheless EMD administration can also increase basal myosin ATPase activity.

In the present study, relaxation of intact cardiac cells was significantly attenuated, particularly at higher (0.3 and 1 μM) OM concentrations but was less affected upon EMD or Levo treatments. This is compatible with the finding of our previous study in which OM also substantially prolonged the relaxation and increased the passive stiffness of permeabilized rat myocyte-sized preparations with a Ca^{2+}-independent mechanism. An impaired diastolic performance - reflected by worsened time constant of isovolumic relaxation (τ) and the rate of the LV pressure decrease (dP/dt_{min}) - was shown earlier after iv. OM administration in rats with volume overload HF during OM treatments. Nevertheless, OM did not impair diastolic function in healthy volunteers at plasma concentrations similar to those applied in this latter study. The possibility that OM may lead to diastolic dysfunction was addressed based on the data collected in the Chronic Oral Study of Myosin Activation to Increase Contractility in Heart Failure (COSMIC-HF) trial. The post hoc analysis showed an increase in isovolumic relaxation time (IVRT) without changes in E/A ratio or E wave. Unfortunately, diastolic function was not assessed in detail in GALACTIC-HF, which would allow further characterization of LV diastolic dysfunction upon oral administration of OM in patients with HFrEF.

Despite having no effects on the intracellular Ca^{2+} transients, OM, EMD and Levo differently alter Ca^{2+}-SL relationships of cardiomyocytes. OM at high concentrations has the most prominent effect on resting SL leading to diastolic cardiomyocyte shortening and prolonged relaxation. EMD also results in a decrease in the resting SL, but without affecting relaxation kinetics. In contrast to OM and EMD, none of these adverse effects were seen during Levo administration. We propose that the widely used clinical parameters of systolic function, such as fractional shortening or ejection fraction can be misleading in case of myosin activators. Here we showed a dramatic decrease in resting SL (which could be paralleled by the reduced

left ventricular diastolic diameter in clinical studies) upon OM administrations. This factor apparently limited fractional shortening at high OM concentrations and might affect ejection fraction calculations as well. The distinct mechanism of OM action on cardiomyocyte resting SL and relaxation kinetics should raise concerns for the clinical administration of the drug and draw our attention to the importance of regular determination of serum OM levels.

5.2 Effects of danicamtiv on systolic and diastolic function *in vivo*

In our *in vivo* study, we have shown by comprehensive echocardiographic analysis that danicamtiv has a significant effect on both systolic and diastolic function in rat hearts. These data and the results of a preclinical study using OM and danicamtiv on S1 myosin of the heart suggest that myosin ATPase rates are increased by direct myosin activators even at diastolic Ca^{2+} levels.

Our current assessment on LV contractility revealed significant increases in FS, SV, EF, and CO upon danicamtiv administration, similarly to previous findings obtained with OM. In contrast to OM, which decreased both EDD and ESD in humans, here we found that danicamtiv decreased only ESD but not EDD in rat hearts. These findings are in accordance with the preclinical data of an experimental canine model of HF, and to some degree also with the results of a clinical phase 2a trial, where EDD appeared somewhat less sensitive for danicamtiv than ESD. In a recent study, where the effects of danicamtiv and OM were compared in a human engineered myocardium, danicamtiv led also to a greater augmentation of systolic contraction with less negative effects on relaxation. Increased LV filling pressure is known to be compensated by an increase in atrial contractile function. In the case of danicamtiv, this phenomenon was well reflected by a significant increase in the transmitral A wave and a decrease in the E wave; thus, a characteristic alteration in the E/A ratio, a major parameter of diastolic function. Accordingly, these changes might be sufficient to compensate for an adequate LV filling. Taken together, our data and that of others implicate higher gains in SV and CO and more favourable diastolic effects for danicamtiv than for OM.

OM treatment was associated with a small decrease in heart rate, which was associated with positive effects due to improved contractile function in the GALACTIC HF trial. In our in vivo experiments, heart rate did not change. The increase in systolic ejection time and systolic contraction time appear as hallmarks of myosin activators, as was shown first for OM treatments. Of note, an increased systolic time results in a shortened diastolic duration, so that the ratio of diastolic to systolic durations will also be affected by danicamtiv treatment, with potential implications for coronary perfusion and troponin release. In contrast to the effects of

OM in humans, the isovolumic contraction time (IVCT) and IVRT were not affected by danicamtiv in rat hearts. Nevertheless, SET was significantly increased by danicamtiv, suggestive for an improvement in the myocardial performance index (Tei index) by danicamtiv.

Here, we evaluated myocardial strain and velocity by using speckle tracking (STE, non-Doppler, or 2-dimensional strain) echocardiography. Strain imaging is a novel approach for assessing myocardial function and has not been included in routine diagnostic methods yet. It is important to note that strain echocardiography, especially in small animals, has also been also less studied, although it provides a very sensitive approach to detect alterations in LV function. Myocardial velocity and strain can be also measured by TDI, though STE 2D-strain is a superior technique since, in contrast to TDI, the obtained results are angle-independent. In general, data obtained by 2D-strain show a good correlation with sonomicrometry and magnetic resonance imaging (MRI), the gold-standard tool of myocardial deformation and volume analysis. Here, danicamtiv treatment was shown to decrease the contraction velocity of the myocardium, which was also confirmed by a significant decrease in a 2D-strain parameter, the peak radial systolic velocity.

In our present study, the effects of danicamtiv on LV systolic function were evaluated not only by standard methods (measuring EF, FS, SV, CO, and aortic velocities) but also by 2D-strain echocardiography. We found significantly decreased GLS and GCS values in danicamtiv-treated rat hearts, further confirming the positive effects of the drug on LV contractility as was also reported for danicamtiv in a phase 2a trial in HFrEF patients and in a dog model of HFrEF.

It is to be acknowledged that extrapolation of our results to the failing heart could be limited by the fact that our investigations were carried out on the cardiac preparations of healthy animals. Nevertheless, our results are in accord with those performed in a canine model of heart failure and in a phase 2a trial on danicamtiv.

Taken together, the cardiac effects of OM and danicamtiv are largely similar: both compounds effectively enhance LV systolic function, although they can also limit LV diastolic function. The data presented here implicate advantages for danicamtiv over OM, since danicamtiv did not affect IVRT or IVCT. Moreover, left atrial contractile function was enhanced at a relatively low danicamtiv concentration. To our knowledge, similar effects have not been reported for OM yet.

Like OM, danicamtiv improves LV systolic function without affecting Ca^{2+} homeostasis. In our study, no atrial arrhythmias, malignant ventricular arrhythmias, or conduction

disturbances were observed, which also supports the fact that danicamtiv is independent of the side effects of conventional positive inotropic agents. It is important to note that in a previous *in vivo* study by our laboratory, where OM treatment was also applied to healthy rats, a so-called alternating electromechanical phenomenon was observed, which was not observed with danicamtiv. Although danicamtiv also affects LV diastolic function, this effect is less striking than when OM is applied. To some extent, this difference may be related to the increase in atrial contractility induced by danicamtiv. In addition, diastolic dysfunction appears as a class effect during the administration of direct myosin activators. Based on our results, regular assessment of LV diastolic function in patients treated with danicamtiv should be a major concern.

5.3 Limitations

The experiments were performed at room temperature and low stimulation rates, in vitro on enzymatically isolated canine cardiac myocytes so some caution is required when extrapolating our results to the human heart. Nevertheless, canine cardiomyocytes have similar cellular electrophysiological properties to human cardiomyocytes.

Since the kinetics of biological processes slow down with decreasing temperature, we chose low-frequency stimulation for experiments under standardised conditions. Similar to our present experiments, in a previous study we observed a reduction in myocardial cell length and relaxation at 1 μM or 2 μM OM concentration at 37 °C using stimulation frequencies of 0.5 Hz, 1 Hz, 2 Hz. In contrast to in vitro intact myocytes, potentially lower levels of myosin activation can be achieved at OM concentrations applied in vivo.

5.4 New scientific observations

- We have shown that omecamtiv mecarbil induces a reduction in sarcomere length in the absence of field stimulation and that it has a calcium-sensitizing effect.
- OM and EMD, despite a similar binding site, differently alter myocardial sarcomere dynamics.
- A specific calcium-sarcomere length relationship is shown for OM. We demonstrated that relaxation takes places after the intracellular calcium levels have restored to resting/diastolic level with increasing OM concentrations.
- In our *in vivo* studies, we were the first to describe the slowing of the contraction kinetics by danicamtiv with the use of strain analysis.
- Diastolic dysfunction is a class effect (in the case of omecamtiv mecarbil and danicamtiv) and an unavoidable side effect of myosin activators. In our experiments

improvement of systolic function was supported by an increase in ejection fraction, fractional shortening and non-invasively measured hemodynamic parameters.

- We confirmed that danicamtiv significantly shortens the diastolic duration, as it prolongs the systolic duration, which raises the risk of coronary perfusion.

6 Summary

In summary, our results show that omecamtiv mecarbil (OM) improves systolic function *in vitro* by reducing systolic sarcomere length and prolonging contractions with unchanged Ca^{2+} transients. At higher OM concentrations, a significant reduction in diastolic sarcomere length, a slowing of kinetic parameters and a significant increase in contraction time are observed. All these changes lead to the development of diastolic dysfunction. EMD, despite a similar molecular point of action, decreases both systolic and diastolic sarcomere lengths, but tends to increase kinetic parameters. Levosimendan enhanced contractions without altering diastolic sarcomere length.

In vivo, danicamtiv also significantly increased left ventricular contractility, left ventricular ejection fraction, fractional shortening, stroke volume and cardiac output. Using modern echocardiographic techniques, an improvement in GLS and GCS and a slowing of contraction kinetics (reduction in radial systolic peak velocity and flattening of M-mode contraction slope) were detected.

The most characteristic echocardiographic marker of myosin activators is a significant prolongation of systolic ejection time (SET) without changes in heart rate, IVCT and IVRT. The prolongation of the systole duration results in a shortening of the diastole duration, which may lead to myocardial ischaemia during the administration of these agents.

In addition to the improvement in systolic function, a deterioration in diastolic function could be expected with myosin activators (class effect). Diastolic dysfunction with danicamtiv is not negligible, therefore prior to and during the treatment its accurate assessment and close monitoring is highly recommended. These potentially adverse effects may significantly limit/question the use of this new class of drugs in clinical practice.

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List of publications related to the dissertation

1. **Ráduly, A. P.**, Tóth, A., Sárkány, F., Horváth, B., Szentandrássy, N., Nánási, P. P., Csanádi, Z., Édes, I., Papp, Z., Borbély, A.: Omecamtiv mecarbil augments cardiomyocyte contractile activity both at resting and systolic Ca2+ levels.
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2. **Ráduly, A. P.**, Sárkány, F., Kovács, M. B., Bernát, B., Juhász, B., Szilvássy, Z., Pórszász, R., Horváth, B., Szentandrássy, N., Nánási, P. P., Csanádi, Z., Édes, I., Tóth, A., Papp, Z., Priksz, D., Borbély, A.: The Novel Cardiac Myosin Activator Danicamtiv Improves Cardiac Systolic Function at the Expense of Diastolic Dysfunction In Vitro and In Vivo: Implications for Clinical Applications.
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List of other publications

3. Bernát, B., Erdélyi, R., Fazekas, L., Garami, G., Szekeres, R., Takács, B., Bombicz, M., Varga, B., Sárkány, F., **Ráduly, A. P.**, Romanescu, D. D., Papp, Z., Tóth, A., Szilvássy, Z., Juhász, B., Priksz, D.: Drug Candidate BGP-15 Prevents Isoproterenol-Induced Arrhythmias and Alters Heart Rate Variability (HRV) in Telemetry-Implanted Rats.
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